3398

Changes in State of Water in Proteinaceous Systems

E. BERLIN, P. G. KLIMAN, AND M. J. PALLANSCH

Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C. 20250

When lyophilized preparations of β-lactoglobulin, bovine serum albumin, and calfskin collagen sorbed at least 0.18 gm H_2O per gram of dried protein, it was observed through differential scanning calorimetry (DSC) that the heat of vaporization of the sorbed water was 80–125 cal/gm higher than the ΔH_v of liquid water. When less H_2O is sorbed, at lower values of P/P_o, the ΔH_v for the sorbed water was equivalent to that of free water. These differences in strength of H_2O-protein binding may be attributed to the availability of protein surfaces or suitable H_2O binding sites. At the higher moisture levels the solid protein matrix has become swollen and possibly conformational changes have occurred in the protein molecules permitting more H_2O-surface contacts and the formation of an "icelike" structure. Accordingly extensive water binding was observed in completely wet systems by measuring the heat of fusion of the water associated with wet pellets of ultracentrifugal casein. Water bound in an "ice" structure will not freeze on cooling to low temperatures (−70°C) and may therefore be assessed through DSC. Such bound water was found to correspond to 50%–60% of the dry weight of the protein present.

The intermolecular structure of liquid water has attracted the attention of both biologists and physical chemists in recent years (1, 2). An area of particular concern in relation to biological systems has been the elucidation of the structure of H_2O at macromolecular surfaces. Though the several models proposed for such H_2O structure vary in detail, there is wide acceptance of the concept of water existing in an "icelike" configuration or some quasi-solid form in the region of dissolved macromolecules.

The sorption of H_2O vapor by dried proteins is generally assumed to involve the binding of H_2O molecules to specific hydrophilic sites at lower relative humidities followed by condensation or multimolecular adsorption as the humidity increases (3, 4). It is conceivable that this bound water may exist in an ordered array on the protein surface forming an "icelike" structure or conversely the H_2O molecules should possibly be considered as rigidly held apart at specific binding sites.

The existence of protein bound H_2O in a quasi-solid configuration should be apparent from measurements of the heat required to effect phase changes in this associated water. One might expect an increase in ΔH_v, the heat required to vaporize this bound water, over that of liquid H_2O. Similarly one would expect ΔH_l, the heat of fusion of the solvent H_2O in an aqueous protein solution, to be affected since bound H_2O, if held in an icelike form, should not freeze. In the present study relevant data consisting of these enthalpies were obtained by differential scanning calorimetry (DSC).

EXPERIMENTAL SECTION[1]

Materials. Bovine casein was isolated by high-speed centrifugation of 230 ml of raw skim milk at 78,410 × g for 20 min in the Spinco Model L preparative ultracentrifuge. The casein pellets were washed twice by dispersing in distilled water to a total volume of

[1] Mention of brand or firm names does not constitute an endorsement by the Department of Agriculture over others of a similar nature not mentioned.

150 ml, by means of a Ten Bröeck tissue grinder, and recentrifuging. The washed pellets were then either used directly in calorimetric studies or redispersed in distilled water and lyophilized for later use in water sorption studies. β-Lactoglobulin was prepared from raw skim milk according to the method of Fox *et al.* (5) and dried by lyophilization. The other proteins were lyophilized samples of commercial preparations of the highest available purity. Bovine serum albumin (BSA) was a preparation of Cohn Fraction V from bovine plasma purchased from Armour Pharmaceutical Corporation. Acid-soluble calfskin collagen A was from Calbiochem and an additional sample of β-lactoglobulin was purchased from Nutritional Biochemicals Corporation.

Calorimetric standards used were: indium, lead, tin (Perkin-Elmer Corporation), benzoic acid (James Hinton, 99.99% zone refined), naphthalene (James Hinton, 99.999% zone refined), and anthracene (James Hinton, 99.999% zone refined).

Methods. The Perkin-Elmer Model DSC-1B differential scanning calorimeter was used to determine the heats of fusion and of vaporization of water associated with these proteins. This involved using the calorimeter in two distinctly different modes of operation with respect to such variables as sample size and preparation, atmosphere control, heating rate, and the temperature interval scanned.

ΔH_v *Studies.* Lyophilized protein samples were used either as received, containing residual moisture from lyophilization, or after adjustment of the moisture content by equilibration against H_2O vapor at different P/P_o levels. Samples were placed in humidostats maintained at 40%, 75%, and 95% relative humidity with appropriate saturated salt solutions (6).

In determining ΔH_v, 3–5 mg samples, weighed with the Cahn Model M-10 electrobalance, were packed in aluminum sample pans and scanned at a programmed heating rate of 10°/min, over a suitable temperature interval to effect complete H_2O desorption. We used either the standard crimped sample pans supplied by the Perkin-Elmer Corporation, which are not hermetically sealed and allow for rapid removal of H_2O vapor from the calorimeter cell in a stream of N_2, or hermetically sealed capsules (Perkin-Elmer volatile sample sealer accessory) with a small pinhole punctured in the lid. The latter sample container restricts vapor removal under increasing pressure, resulting in completion of the desorption at a higher temperature.

Precautions are necessary in such dehydration studies to avoid transfer of water vapor between the sample and the atmosphere prior to scanning in the calorimeter. This was accomplished by rapidly cooling the sample, in either type of container, to 0°C in the calorimeter cell prior to beginning the scan. Loss of moisture from the sample is negligible at this temperature and additional H_2O uptake would not occur in the flowing N_2 atmosphere.

ΔH_l *Studies.* In these determinations samples of wet protein were packed in sealed capsules and rapidly cooled in the calorimeter cell to −40°C to freeze the solvent H_2O. The samples were then heated at a programmed rate of 1.25°/min to 20°C and the energy required to melt the solvent H_2O was measured.

Estimation of bound water was based on the premise that such H_2O would not freeze even at extremely low temperatures. The observed heat requirement for melting the ice was compared with that which should be needed to melt the total H_2O present in the system and the observed discrepancy was attributed to water binding. On the basis of a ΔH_l value for pure H_2O of 79.6 cal/gm it is then possible to compute the mass of such bound H_2O per unit mass of protein on a dry weight basis.

For these studies segments of washed casein pellets as obtained upon centrifugation and samples of the other proteins dispersed in a minimal amount of H_2O were used. The total mass of protein and solvent was usually 3–5 mg. The total H_2O content in each capsule was determined subsequent to the calorimetric procedure by puncturing the capsule and drying the sample under vacuum.

Variables tested in these experiments included the effects of: (1) washing the protein with distilled H_2O; (2) repeating the freezing and thawing sequence on the same sample

several times; and (3) freezing the protein suspension at various temperatures as low as −70°C.

In all scans with the calorimeter no diluents were used other than water, and empty containers and covers identical to those of the samples were placed in the reference pan of the DSC-1B.[2] During a calorimetric run the DSC-1B measures the energy required to maintain the sample and reference pans isothermal with respect to each other while both are heated or cooled at an identical programmed rate. An electrical signal proportional to this energy requirement is displayed on a strip chart recorder. When a thermal event occurs the excursion of the recorder pen is proportional to the energy required, resulting in a curve or peak the area of which can then be correlated with this energy in calories.

The temperature scale of the DSC-1B was calibrated as directed in the manufacturer's instruction manual using the melting points of high-purity samples of indium, lead, and tin. Calibration of the electrical output signal from the calorimeter for quantitative evaluation of the recorder tracings was performed by correlating the area under the endothermic peak for the melting of a pure sample of indium metal with its known heat of fusion (ΔH_l = 6.79 cal/gm). Peak areas were measured with a polar planimeter (Keuffel and Esser Company, K and E 4236). Measurements of ΔH_l for benzoic acid, naphthalene, and anthracene were made to check the DSC-1B and its calibration. These materials were scanned in sealed capsules to avoid errors from sublimation. The ΔH_l values were within experimental error of those found by Hampson and Rothbart (7) and other values from the literature as tabulated by them.

Values of ΔH_v were computed by measuring the areas of the endothermic peak for this dehydration and vaporization process and correlating the quantity of absorbed heat with the mass of desorbed H_2O. The H_2O desorption values were obtained by weighing the samples before and after the DSC run. Mass values obtained by this procedure were in agreement with parallel measurements of H_2O content by evacuation at ambient temperature and thermogravimetric analyses (TGA).

The supplementary thermogravimetric analyses were carried out with the Cahn RG recording electrobalance together with the "Little Gem" TGA accessory of the Cahn Instrument Company and a Varian Model 100 X-Y recorder. Samples were heated in a platinum bucket from ambient temperature (23–27°C) to 200°C at a rate of 5°/min.

Evaluation of the melting peaks for ΔH_l was performed according to a published (8, 9) method for purity determination which accounts for influences of freezing point depression and the difficulties of accurately determining the melting range from DSC traces for such mixtures. In this method a plot of T_s *vs.* $1/F$ is made, where T_s is the instantaneous temperature of the sample during the scan and F represents the fraction of sample melted at the same time. The value for F is obtained from the ratio of the partial area from the beginning of the peak until T_s to the total area under the peak. With a pure material the plot of T_s *vs.* $1/F$ is linear but when the substance is not pure, *i.e.*, less than 99.5 mole per cent, the points may not fall on a straight line. The nonlinearity is attributed to missing some of the peak area before the instrument departs measurably from the baseline owing to the noise level and sensitivity involved. The plot is then normalized through trial-and-error additions in small increments to both the partial and total areas. Application of this procedure insures the inclusion of the total requisite area for the ΔH_l calculations, thus avoiding what might be a significant source of error.

RESULTS

Values for ΔH_v as calculated from the area under an endothermic peak attributed to sorbed H_2O vaporization, are given for the various proteins in Table I. The origin of this peak was verified by its absence when protein samples previously dried under vacuum were examined in the DSC-1B. The endotherm under consideration was very

[2] In this paper we shall use the abbreviation DSC to refer to the method of differential scanning calorimetry and DSC-1B to refer to the Perkin-Elmer instrument.

TABLE I

HEATS OF VAPORIZATION ΔH_v OF WATER SORBED BY DRIED PROTEINS

Protein	Grams H_2O/ gram protein	ΔH_v (*cal/gm*)
Casein	0.096	560
	0.178	571
	0.187	678
	0.208	680
	0.238	626
	0.366	629
	0.381	636
Collagen	0.134	594
	0.240	675
	0.280	674
	0.348	718
β-Lactoglobulin	0.090	569
	0.125	560
	0.157	530
	0.213	677
	0.220	676
Bovine serum albumin	0.055	667
	0.119	569
	0.162	576
	0.178	628
	0.214	651
	0.453	671

TABLE II

EXTENT OF WATER BINDING BY PROTEINS WHEN DISPERSED IN WATER

Sample no.	Protein (*mg*)	H_2O total (*mg*)	ΔQ (*mcal*)	$\frac{gH_2O \text{ bound}}{g \text{ protein}}$
		Casein		
1	1.436	3.674	224.7	0.591
2	1.762	4.710	303.9	0.507
3	1.908	4.940	331.8	0.509
4	0.792	5.198	291.0	0.578
5	1.896	4.712	257.9	0.557
6	1.962	4.348	268.4	0.537
7	1.942	4.316	257.9	0.598
8	1.924	4.724	291.8	0.550
				Av 0.553
		Bovine Serum Albumin		
1	3.224	2.928	103.3	0.506
2	3.158	3.208	129.5	0.501
3	3.212	3.246	135.1	0.482
4	3.280	3.560	162.5	0.463
				Av 0.488
		β-Lactoglobulin		
1	2.446	4.586	260.1	0.539
2	2.396	4.474	254.5	0.533
3	2.396	4.696	262.0	0.586
4	2.496	4.626	259.0	0.560
5	2.272	4.412	242.4	0.549
				Av 0.553

broad with a range between the limits of 5° or 10°C and 150° to 185°C. Though some variations in temperature range were observed, in most instances the endotherms ranged over the interval between 10° and 160°C with peak maxima at 80° to 90°C.

At lower to intermediate quantities of sorbed water the values for ΔH_v are close to that of pure H_2O, for which we found a value of 550 ± 23 cal/gm as the average for fifteen separate determinations. At higher moisture levels, when the sorbed water exceeded 0.18 gm H_2O per gram of protein, the ΔH_v values exceeded that of pure H_2O . These differences were clearly not within experimental error. On the other hand at low moisture levels, as when 0.05 gm H_2O are sorbed per gram of dry bovine serum albumin, a higher value of ΔH_v = 667 cal per gram of H_2O was observed.

Data showing the extent of water binding by casein, β-lactoglobulin, and bovine serum albumin when in aqueous solution or dispersion are given in Table II. The proteins apparently bind a quantity of water equivalent to approximately 50% of their dry weight. The same magnitude of water binding was observed with unwashed casein; however, these data differed from those of the washed pellets in the nature of the T_s *vs.* $1/F$ plots. A normalization procedure was required with the unwashed casein whereas none was needed after washing. This is to be expected as very little low molecular weight material should be present after washing.

The data in Table II were obtained with samples frozen at −40°C; however, identical data were obtained when the solutions were frozen at −70°C. Repeated freezing and thawing cycles apparently did not alter the extent of H_2O binding as measured in these experiments.

DISCUSSION

Published DSC studies of water binding in biological systems are few, including essentially the thesis of Karmas (10), who em-

ployed the DSC-1B in studying solutions of amino acids and two natural protein systems—egg albumin and beef muscle—and the work of Ladbrooke *et al.* (11), who studied the physical structure of myelin and so obtained information pertaining to the organization of lipids and water in the myelin structure. In a previous paper Chapman *et al.* (12) studied H_2O phospholipid binding using DSC and considering bound H_2O as unfreezable water.

A number of reports, however, have been published on the application of the related technique of differential thermal analysis (DTA) to the study of water binding by such proteins as silk, keratin, collagen, and bovine, porcine, and human blood albumins as well as a number of synthetic polypeptides (13–17). The DTA curves differed from our results in that the H_2O desorption endotherms were reported to extend over the interval between 100° and 200°C with peak maxima between 125° and 150°C, generally temperatures higher than those we observed with the DSC-1B, particularly when crimped sample containers were used. Karmas (10) also observed rapid evaporation of H_2O at lower temperatures, and he has discussed the differences between the DSC and DTA work in terms of the geometry of the DTA cell, pointing out the restriction of vapor egress from the DTA cell. Hence, using a sealed capsule with a pinhole as in the present study should lead to better protection of the sample prior to heating in the DSC-1B, as well as slightly elevating the temperature range for the desorption process.

Most published papers on DTA of proteins do not include any values for the ΔH_v of the bound water; however, Puett (17) has reported a value of 614 cal per gram of water removed from crosslinked collagen, and more recently Hoyer and Birdi (15) have reported 616 cal per gram of H_2O for bovine albumin. Puett stated that generally for proteins and polypeptides the ΔH_v of bound water exceeded that of free water by some 70 to 100 cal/gm. These results are in close agreement with our data at higher values of sorbed water. Both Puett and Hoyer and Birdi gave protein data only where at least 0.18 gm H_2O were bound per gram of protein and neither studied the effect of the amount of sorbed H_2O on ΔH_v.

In the present study we have shown a definite variation in ΔH_v as a function of the amount of sorbed water, with an increase in ΔH_v occurring after approximately 0.18 gm H_2O are sorbed per gram of dry protein (Table I), reflecting a change in the energy through which H_2O is bound to the protein. This is somewhat unexpected as generally physical adsorption is accompanied with a decreasing heat of adsorption approaching the heat of liquefaction of the sorbate with increasing surface coverage.

The results of the present study may be explained in terms of a mechanism of H_2O sorption by dry protein. At low humidities, H_2O is bound to specific hydrophilic sites through hydrogen bonding, usually resulting in a heat of adsorption equivalent to 12 kcal/mole; however, as more water is sorbed the binding is weaker and the heat of adsorption decreases. The adsorption of H_2O vapor by a protein also leads to swelling of the solid, resulting in increased available surfaces as more H_2O is sorbed and the protein swells. It is conceivable that at higher P/P_o values the swelling has proceeded to a sufficient extent to allow more complete hydration of the protein with the sorbed water forming an "icelike" or quasi-solid structure, as is postulated for water in the vicinity of dissolved macromolecules. This would result in ΔH_v for the sorbed water being closer to the heat of sublimation of ice than to the heat of vaporization of liquid water. This is shown to be so with the data reported here for ΔH_v when the sorbed H_2O exceeded 0.18 gm H_2O per gram of protein. Thus, once a critical amount of H_2O is sorbed and the protein can possibly undergo a change in conformation this water may be considered as held in an "icelike" structure. Additional bound water would then yield similar higher ΔH_v values, and the amount of water sorbed at the saturation pressure should approach the amount of H_2O bound in an icelike layer in solution. The presence of 0.5 gm bound water per gram protein in solution as reported here (Table II) is in agreement with this interpretation as the hydration of solid protein reportedly exceeds

0.5 gm H_2O per gram of protein as P/P_o approaches unity (18).

The conclusions reached in this paper are based on enthalpy measurements only; however, in considering the state of bound water, a knowledge of the entropy change in the sorption process would be useful. Values for ΔS cannot be computed from our DSC data. The binding of individual H_2O molecules to specific sites on the protein surface and certainly the "freezing" of H_2O into an "ice structure" should yield negative ΔS values owing to the increased order in the system. Sinanoglu and Abdulnur (19) have estimated the entropy decrease of water in the vicinity of biopolymers due to structuring of H_2O from experimental data on the formation of gas hydrates, changes in crystal structure of ice, and differences in the surface entropies of water-air and water-hydrocrabon interfaces. The empirical decrease in entropy of water (20) when a nonpolar solute is introduced implies an increase in local water structure and fits in well with hydrogen-bonded cluster theories of the structure of liquid water (1, 21) and has led to the concept of the hydrophobic bond (20). Frank and Evans (22) introduced the concept of a special iceberg structure with more hydrogen bonding than in bulk water to explain the entropy data, and Nemethy and Scheraga (23) have developed a detailed model based on this concept. Though viewpoints concerning the detailed nature of bound water near dissolved proteins vary between the "surface solvation" concepts of Kauzmann (20) and the ice structure viewpoint of Klotz (24, 25), there is agreement that binding of H_2O to biopolymers in solution implies an entropy loss and structuring of the water.

Davis and McLaren (26) have computed ΔS values for the sorption of water vapor by dried proteins from data previously reported by Bull (27). Negative ΔS values were computed for silk, wool, collagen, and crystalline β-lactoglobulin at all P/P_o values whereas positive ΔS values were obtained for salmine, lyophilized β-lactoglobulin, and lyophilized or heat-coagulated egg albumin in certain P/P_o ranges. These authors concluded that H_2O sorption on proteins involves two entropic contributions: a negative ΔS for the localization of the H_2O and a positive ΔS due to incipient solution formation, *i.e.*, structural transformations in the protein representing the inception of the solution process. Recently Bull and Breese (18) have reported negative ΔS values at all levels of P/P_o for H_2O sorption on ovalbumin.

The conclusions of Davis and McLaren (26) are not necessarily at variance with ours as incipient solution formation should be considered as a shift in the configuration of the protein ($+\Delta S$) allowing the water to be associated with the protein in a manner similar to that occurring in solution which involves structured water in some form, according to all present views of protein hydration.

REFERENCES

1. Kavanau, J. L., "Water and Solute-Water Interactions." Holden-Day, San Francisco, (1964).
2. Berendsen, H. J. C., *in* A. Cole, ed, "Theoretical and Experimental Biophysics," p 1. Marcel Dekker, New York, (1967).
3. Pauling, L., *J. Amer. Chem. Soc.* **67**, 555 (1945).
4. McLaren, A. D., and Rowen, J. W., *J. Polym. Sci.* **7**, 289 (1951).
5. Fox, K. K., Holsinger, V. H., Posati, L. P., and Pallansch, M. J., *J. Dairy Sci.* **50**, 1363 (1967).
6. Rockland, L. B., *Anal. Chem.* **32**, 1375 (1960).
7. Hampson, J. W., and Rothbart, H. L., *J. Amer. Oil Chem. Soc.* **46**, 143 (1969).
8. Perkin-Elmer Thermal Analysis Newsletter No. 5 and 6 (1966).
9. Driscoll, G. L., Duling, I. N., and Magnotta, F., *in* R. S. Porter and J. F. Johnson, eds, "Analytical Calorimetry," p 271. Plenum Press, New York, 1968.
10. Karmas, Endel, Ph.D. Thesis, Rutgers-The State University, New Brunswick, New Jersey, 1968.
11. Ladbrooke, B. D., Jenkinson, T. J., Karmat, V. B., and Chapman, D., *Biochim. Biophys. Acta* **164**, 101 (1968).
12. Chapman, D., Williams, R. M., and Ladbrooke, B. D., *Chem. Phys. Lipids* **1**, 445 (1967).
13. Crighton, J. S., and Happey, F., *in* G. N. Ramachandran, ed, "Conformation of Biopolymers," p 623. Academic Press, London and New York, 1967.
14. Felix, W. D., McDowall, M. A., and Eyring, H., *Text. Res. J.* **33**, 465 (1963).
15. Hoyer, H. W., and Birdi, K. S., *Biopolymers* **6**, 1507 (1968).

16. Morita, H., *Biopolymers* **4**, 215 (1966).
17. Puett, D., *Biopolymers* **5**, 327 (1967).
18. Bull, H. B., and Breese, K., *Arch. Biochem. Biophys.* **128**, 488 (1968).
19. Sinanoglu, O., and Abdulnur, S., *Fed. Proc.* **24**, *Suppl.* **15**, S-12 (1965).
20. Kauzmann, W., *Advan. Protein Chem.* **14**, 1 (1959).
21. Frank, H. S., *Fed. Proc.* **24**, *Suppl.* **15**, S-1 (1965).
22. Frank, H. S., and Evans, M. W., *J. Chem. Phys.* **13**, 507 (1945).
23. Nemethy, G., and Scheraga, H. A., *J. Chem. Phys.* **36**, 3382 (1962).
24. Klotz, I. M., *Science*, **128**, 815 (1958).
25. Klotz, I. M., *Fed. Proc.* **24**, *Suppl.* **15**, S-24 (1965).
26. Davis, S., and McLaren, A. D., *J. Polym. Sci.* **3**, 16 (1948).
27. Bull, H. B., *J. Amer. Chem. Soc.* **66**, 1499 (1944).